***Schistosoma bovis*: plasminogen binding in adults and the identification of plasminogen-binding proteins from the worm tegument.**

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Abstract

Schistosoma bovis is a ruminant haematic parasite that lives for years in the mesenteric vessels of the host. The aim of this work was to investigate the ability of adult *S. bovis* worms to interact with plasminogen, a central component in the host fibrinolytic system. Confocal microscopy analysis revealed that plasminogen bound to the tegument surface of the male –but not female- *S. bovis* worms and that this binding was strongly dependent on lysine residues. It was also observed that a protein extract of the worm tegument (TG) had the capacity to generate plasmin and to enhance the plasmin generation by the tissue-type plasminogen activator. Proteomic analysis of the TG extract identified 10 plasminogen-binding proteins, among which the major ones were enolase, glyceraldehyde-3-phosphate dehydrogenase and actin. This study represents the first report about the binding of plasminogen to *Schistosoma sp.* proteins.

Key words: *Schistosoma bovis*; Tegument; Plasminogen binding; Proteome; Mass spectrometry; Confocal microscopy.

1. Introduction

In many tropical and subtropical countries, schistosomes are one of the major causes of disease in humans and domestic animals (De Bont and Vercruysse, 1998; Mahmoud, 2001). *Schistosoma bovis* is a ruminant haematic parasite that lives in the portal mesenteric system of the host. It belongs to the same species complex as the human pathogen *S. haematobium* and indeed is an immunological analogue of *S. haematobium* (Agnew et al., 1989), which makes studies of *S. bovis* an attractive goal from the perspectives of both veterinary and comparative medicine.

Like human schistosomes, adult *S. bovis* worms live for years in the blood vessels of their definitive host without being removed by the host defensive systems (Pearce and MacDonald, 2002). It is known that schistosomes trigger potent immune responses in their hosts, but it is also apparent that they have evolved multiple strategies to evade the host immune defence mechanisms (Abath and Werkhauser, 1996). It is reasonable to assume that in this intravascular habitat the parasite must interact not only with the immune system but also with the haemostatic system of the host. Notwithstanding, relatively few studies have investigated the interactions between schistosomes and elements of host haemostatic defence mechanisms. These studies have essentially focused on the interaction between the parasite and platelets (DeMarco et al., 2003; Stanley et al., 2003). In this sense, it has been observed that all developmental stages of *S. mansoni*, except the eggs, express ATPase activity on their tegument surface and that this ATPase activity would inhibit platelet activation (DeMarco et al., 2003). It has been also reported that infection with *S. mansoni* induces immune-dependent thrombocytopaenia (Stanley et al., 2003) and that this thrombocytopaenia could partially explain why blood clots do not form around adult worms (in Ruppel et al., 2002).

Another mechanism that could help to prevent clot formation could be activation of the fibrinolytic system, in which plasminogen plays a key role.

Plasminogen is a single-chain glycoprotein with a molecular mass of about 92 kDa and it represents the monomeric proenzyme of the serine protease plasmin, which degrades fibrin and extracellular matrices (Plow et al., 1995). The binding of plasminogen to mammalian and

bacterial cells is mediated by its five kringle domains, which have affinity for lysine residues, and in fact almost all plasminogen-receptor proteins so far identified have carboxy-terminal lysine residues (Plow et al., 1995).

Interactions with plasminogen have been observed in bacteria, fungi, protozoa and helminths. The interactions of some bacterial and fungal pathogens with the plasminogen-plasmin system have been the most studied, and in the case of group A Streptococci, *Staphylococcus aureus*, *Yersinia pestis*, *Borrelia burgdorferi* and *Candida albicans* the interaction with plasminogen has been shown to be involved in invasiveness within the host (Goguen et al., 2000; Lähteenmäki et al., 2001; Crowe et al., 2003). The protozoa *Leishmania mexicana* and *Trypanosoma cruzi* have been reported to bind plasminogen and also to enhance the activation of plasminogen by tissue-type plasminogen activator (t-PA) (Avilan et al., 2000; Almeida et al., 2004). In helminths, two plasminogen-binding proteins have been identified: enolase in *Fasciola hepatica* and *Onchocerca volvulus* and glyceraldehyde-3-phosphate dehydrogenase in *O. volvulus* (Jolodan et al., 2003; Bernal et al., 2004; Erttmann et al., 2005).

Since adult *S. bovis* worms live for years in the blood vessels of the host, it is reasonable to assume that this parasite would interact with the plasminogen-plasmin system as a way to prevent blood clotting around it. The parasite molecules that could potentially bind plasminogen would be those that the parasite exposes to the host during infection, such as the proteins expressed on its tegument surface.

Accordingly, the aims of this study were to investigate whether plasminogen binds to the tegument of adult *S. bovis* worms and to identify the proteins most likely involved in such binding.

Here, we demonstrate that plasminogen binds to the surface of male-but not female-worms and that an extract of tegument proteins induces the generation of plasmin on its own and also enhances the plasmin generation induced by t-PA. Finally, by mass spectrometry analysis, we identified in that extract 10 plasminogen-binding proteins, among which the biologically relevant receptors are presumably present.

2. Materials and methods

2.1. Maintenance of the *S. bovis* life cycle

A strain of *S. bovis* from Salamanca (Spain) was maintained in the laboratory in its natural hosts: *Planorbarius metidjensis* snails and sheep. Each snail was infected with five miracidia from eggs obtained from experimentally infected sheep faeces. The sheep were infected percutaneously with 2000 *S. bovis* cercariae by submerging a fore-limb for 30 min in a suspension of these cercariae. At 6 months post-infection, the sheep were sedated with ketamine (10 mg/kg) and then sacrificed by bleeding through the jugular vein. Adult *S. bovis* worms were recovered by dissection of the mesenteric vessels from the entire gut. The viability of the worms was examined microscopically, after which intact parasites were processed immediately to obtain the tegument extracts or for the analyses by confocal microscopy.

2.2. Preparation of tegument protein extract

A tegument protein extract (TG) was prepared from live adult worms according to the following procedure. The parasites were washed thoroughly in Tris-buffered saline (TBS) and incubated in 1% Triton X-100 in TBS at 4°C with gentle shaking for 30 min. Following this, the incubation medium was recovered and a cocktail of protease inhibitors was added, as indicated by Maizels et al. (1991). After that, the extract was clarified by centrifugation at 10000xg, dialyzed against water for 24 h, and concentrated by vacuum centrifugation to a concentration of > 3 µg/µl. The protein concentration of the TG extract was measured by the method of Markwell et al. (1978).

2.3. Plasminogen binding to tegument proteins

To determine whether plasminogen would bind to the TG extract, an ELISA was performed. Multiwell plates (Sigma) were coated with 1 µg of TG protein per well diluted in carbonate buffer, pH 9.6, overnight at 4°C. Non-specific binding sites on the plates were blocked by incubation with 1% BSA in PBS (PBS-BSA). After washing with PBS containing

0.05% Tween 20, the wells were incubated with increasing amounts (from 0 to 6 µg) of human plasminogen (Acris antibodies) diluted in PBS-BSA. The wells were washed again and then incubated with 0.1 µg/well of a peroxidase-conjugated goat anti-human plasminogen IgG (Cedarlane Laboratories). All incubations were performed over 1 h at 37 °C. Ortho-phenylenediamine was used as a chromogen substrate for peroxidase. In parallel, competition experiments were carried out by including 40 mM of the lysine analogue ε-aminocaproic acid (εACA) during plasminogen binding. All the multiwell plates included some control wells that were coated with BSA only (without TG).

2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The TG extract was subjected to SDS-PAGE in 12% polyacrylamide gels according to the method described by Laemmli (1970) in a Mini Protean cell (Bio-Rad). Running proceeded at 15 mA/gel for the first 15 min and then at 30 mA/gel. After running, the gels were either stained with silver stain or electroblotted onto nitrocellulose membranes for ligand blot analysis.

2.5. Two-dimensional electrophoresis (2DE)

Isoelectric focusing (IEF) was performed in 7-cm immobilised pH gradient (IPG) strips (Bio-Rad) with linear pH ranges of 5-8 and 7-10, at 50 µA/strip, using a Protean IEF Cell (Bio-Rad). As observed by Pérez-Sánchez et al. (2006), in these pH ranges the TG components can be adequately resolved.

The TG samples, containing 25 µg for 2D silver-stained gels or 40 µg for 2D Western blotting, were diluted in 125 µl of rehydration buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM dithiothreitol (DTT), 0.2% IPG buffer of the respective pH gradient, and bromophenol blue) and allowed to mix gently for 1 h at room temperature before centrifugation for 30 min at 18000xg to remove all particulate material. Supernatants were applied to the IPG strips by in-gel rehydration at 20 °C for at least 12 h, after which IEF were run for a total of 20000 Vh.

After IEF, the strips were reduced in equilibration buffer (6 M urea, 0.05 M Tris, pH 8.8, 2% SDS and 20% glycerol) containing 2% DTT for 15 min and were then alkylated in equilibration buffer containing 2.5% iodoacetamide for 10 min. The second dimension was performed in 12% polyacrylamide gels.

The 2D gels were stained with a mass spectrometry compatible silver stain (Stochaj et al., 2003) and scanned using an Image Scanner (GE Healthcare).

2.6. Ligand blotting

Proteins were electrotransferred from 1D or 2D gels to nitrocellulose membranes at 400 mA for 90 min. The membranes were blocked with 3% BSA in TBS for 1 h at 37° C, rinsed 3 times with washing buffer (TBS containing 0.05% Tween 20, TBST), and incubated overnight at 4° C with 35 µg/ml of human plasminogen diluted in TBST-25 mM EDTA-1% BSA. After four new washes, the blots were incubated for 1 h at 37° C with 0.5 µg/ml of the peroxidase-conjugated goat anti-human plasminogen IgG diluted in TBST-1% BSA. The 1D ligand blots were revealed with 4-chloro-1-naphthol. The 2D ligand blots were revealed with the ImmunoStar HRP Chemiluminescent Kit (Bio-Rad) and their images were acquired with the Fluor-S Multimager system (Bio-Rad). Negative controls were also included in which the TG or the plasminogen had been omitted. These assays were performed three times.

2.7. Image analysis

Analysis of 2D gels and 2D Western blots was accomplished using ImageMaster 2D Platinum Software v5.0 (GE Healthcare). The 2D Western blots and their homologous silver-stained gels were aligned to isoelectric points (pI) and molecular weights (MW) and then matched by ImageMaster software in order to identify the plasminogen-binding spots in the gels. Predicted matches were also verified visually.

2.8. Mass spectrometry (MS) and protein identification

These analyses were carried out as described in Pérez-Sánchez et al. (2006). Protein spots were excised manually and then digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics). The digestion protocol used was that of Schevchenko et al. (1996). For peptide mass fingerprinting and LIFT TOF/TOF (Suckau et al., 2003) spectra acquisition, an aliquot of α -cyano-4-hydroxycinnamic acid in 33% aqueous acetonitrile and 0.1% trifluoroacetic acid was mixed with an aliquot of the above digestion solution and the mixture was deposited onto an AnchorChip MALDI probe (Bruker-Daltonics).

Peptide mass fingerprint spectra were measured on a Bruker Ultraflex TOF/TOF MALDI mass spectrometer (Bruker-Daltonics) (Suckau et al., 2003) in positive ion reflector mode. Mass measurements were performed automatically through fuzzy logic-based software or manually. Each spectrum was calibrated internally with mass signals of trypsin autolysis ions to reach a typical mass measurement accuracy of ± 25 ppm. The measured tryptic peptide masses were transferred by means of the MS BioTools program (Bruker-Daltonics) as inputs to search the NCBI nr database using Mascot software (Matrix Science). When necessary, MS/MS data from the LIFT TOF/TOF spectra were combined with PMF data for database searches with a parent ion mass tolerance of 20 – 40 ppm and a fragment ion mass accuracy of 0.25 – 0.50 Da.

2.9. Plasminogen activation assays

Plasminogen activation was performed by measuring the amidolytic activity of generated plasmin in a test volume of 100 μ l. Plasminogen (5 μ g) was incubated at room temperature in PBS containing 0.6 mM plasmin substrate S2251 (Sigma) in the absence or presence of increasing amounts of TG (1-12 μ g). Plasminogen activation was initiated by the addition of 10 nM t-PA. In parallel assays, the generation of plasmin in the absence of t-PA was determined. Hydrolysis of the chromogenic substrate was monitored by measuring absorbance at 405 nm.

*2.10. Plasminogen binding on the surface of *S. bovis* worms*

Intact parasites, some of them fixed in 10% buffered formalin solution for 24 h and other unfixed and only washed with PBS were incubated for 2 h at room temperature with 10 µg/ml of human plasminogen diluted in PBS containing 1% BSA. After four washes with PBS, they were incubated with 10 µg/ml of fluorescein isothiocyanate (FITC)-labelled anti-human plasminogen antibody conjugated overnight at 4°C in the darkness. The worms were washed four times with PBS and analyzed by confocal microscopy with a Leica TCS NT microscope, using Argon 488 laser and Long Pass 515 filter. Adult schistosomes were analysed with a 10x Plan Floutar 0.3 NA dry objective and 5 µm step sizes. Competition experiments were carried out by including 40 mM of εACA during plasminogen binding. In parallel, control experiments were done in which the plasminogen, the anti-plasminogen antibody, or both were omitted.

3. Results

3.1. Plasminogen binding to tegument proteins (ELISA and ligand blotting)

By ELISA we observed that plasminogen bound to TG proteins and that the amount of plasminogen that bound to 1 µg of TG protein increased with the amount of plasminogen added to the wells. In control wells, coated only with BSA, we observed a certain degree of non-specific binding of plasminogen (Fig.1), which, however, was not observed in the 1D and 2D ligand blots (see below). In the competition experiments, the inclusion of 40 mM εACA inhibited plasminogen binding to TG. In these wells, the reactivity was identical to that observed in the control wells (Fig. 1).

In order to initiate the identification of the plasminogen-binding proteins, TG extracts were analysed by SDS-PAGE and by ligand blotting with plasminogen. Silver staining of the TG extract showed up the complexity of the protein composition of the extract and revealed at least 24 bands, of different intensities, with MWs between 17 and 116 kDa (Fig. 2).

Ligand blotting revealed at least 13 plasminogen-binding bands with MWs between 31 and 94 kDa. In the control strips, without TG or without plasminogen, no bands were revealed (Fig. 2).

3.2. 2D analysis of TG extracts and identification of plasminogen-binding proteins by mass spectrometry

Optimal separation of TG proteins in 2D-electrophoresis was achieved using 2D gels in two pH ranges, 5-8 and 7-10. In these gels, TG proteins were resolved into approximately 450 spots, with *pIs* between 5.5 and 9.6 and MWs between 15 and 144 kDa (Fig. 3A, 3B). In order to identify the plasminogen-binding spots, gels similar to the previous ones were transferred to nitrocellulose and subjected to ligand blotting with plasminogen. As shown in Figs. 3C and 3D, many spots with MWs between 29 and 88 kDa and *pIs* of 5.9-8.8 were revealed. In the control blots, in which plasminogen incubation was omitted, the anti-plasminogen antibody did not reveal any spots (not shown).

Matching the ligand blots with their homologous silver-stained gels allowed us to localise most of the spots that contained plasminogen-binding proteins in the 2D gels. We selected and cut 30 plasminogen-binding spots for MS analysis: 16 spots were cut from 2D gels in the 5-8 pH range (Fig. 3A), while the remaining 14 were cut from 2D gels in the 7-10 pH range (Fig. 3B).

Table 1 shows the results of the MS analyses. We identified 24 spots, corresponding to 10 proteins. The proteins identified were enolase, actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ATP:guanidino kinase, fructose bisphosphate aldolase, phosphoglycerate mutase, triose-phosphate isomerase, adenylate kinase and two additional proteins similar to two hypothetical proteins of *S. japonicum*. All these proteins were identified by their similarity to the homologous proteins of other species of *Schistosoma* (*S. mansoni* and *S. japonicum*).

The spots most intensely revealed in the ligand blots corresponded to different isoforms of actin, enolase and GAPDH. We identified 8 isoforms of enolase; 4 of actin; 3 of GAPDH,

and 3 of ATP:guanidino kinase. The rest of the proteins were only identified in one spot each. The tryptic peptides of spots 1 and 16 matched the hypothetical proteins of *S. japonicum*. Spot 1 is annotated as glucose-6-phosphate 1-dehydrogenase (Swiss-Prot/TrEMBL database), while owing to its similarity to an mRNA sequence of clone ZZD114 of *S. japonicum* (GenBank accession no. *AY223026*), spot 16 may belong to the enzyme glioxalase I.

3.3. Plasminogen activation by the TG extract

We investigated whether the TG extract was able modify plasminogen activation by a physiologic activator and whether its components had the ability to generate plasmin on their own. To do so, increasing amounts of TG were added to a mixture of plasminogen and t-PA, and plasmin generation was measured. As can be seen in Fig. 4, the addition of TG (1-12 µg) enhanced activation by t-PA as compared to the activation obtained in the absence of TG (0 µg). Additionally, plasmin activity was detected in the absence of plasminogen activator, indicating that TG is capable of activating plasminogen on its own.

3.4. Plasminogen binding on the tegument surface

The tegument surface localisation of plasminogen ligands was determined by incubating intact adult worms with plasminogen and visualizing the binding by incubation with a FITC-labelled anti-plasminogen antibody, followed by confocal microscopic analysis and image sectioning. This study was performed with worms fixed in formalin and with unfixed worms, the same results being obtained in both cases. Projected images and sections clearly showed that plasminogen bound to the surface of adult males (Fig. 5). Plasminogen binding was observed on the ridges of the tegument along the whole of the male body. This binding was more intense on the anterior end, around the oral sucker (Fig 5 A, B, D and E). No plasminogen binding at all was observed in the female worms.

In the competition experiments we observed that plasminogen binding was completely inhibited by 40 mM of ϵ ACA, indicating that lysine-binding sites of plasminogen are involved in this interaction (Fig. 5 C, F).

In the control experiments, in which incubation with plasminogen, with the antibody, or with both was omitted, no fluorescence signal was observed (data not shown).

4. Discussion

The aim of the present work was to investigate the interactions of *S. bovis* with host haemostatic mechanisms; in particular the fibrinolytic system. The study was carried out with intact adult worms and with an extract (TG) that contained the proteins expressed by the adult worms on their tegument surface. These proteins, which are exposed to the host during infection, could interact with plasminogen, a plasma glycoprotein that is the central component of the fibrinolytic system.

Here we demonstrate that adult *S. bovis* worms express plasminogen-binding proteins in their tegument. For plasminogen binding to be biologically relevant, these plasminogen-binding proteins must be exposed on the surface of the worm. Immunofluorescence studies and later confocal microscopy revealed the plasminogen effectively bound to the tegument surface and that this only occurred in the males, and not in the females. In addition, the plasminogen binding on the surface of the tegument was strongly dependent on lysine residues, as is also the case for other fungal, bacterial, protozoan and human systems (Boyle and Lottenberg, 1997; Avilan et al., 2000; Crowe et al., 2003; Almeida et al., 2004). The fact that the females do not bind plasminogen can be explained in terms of the notion that they live in the gynaecophoric canal of the males and hence, at least in principle, would not need to develop mechanisms to prevent the formation of clots on their surface.

In this study it is also shown that the TG extract increases plasmin generation by t-PA, in a similar way to that observed in other parasites such as *Trypanosoma cruzi* and *Leishmania mexicana* (Avilan et al., 2000; Almeida et al., 2004;). We do not know the mechanism by which the TG molecules exert this enhancing effect. Notwithstanding, it is known that the

interaction of plasminogen with certain molecules results in large conformational changes that enhance the ability of t-PA to generate plasmin (Anglés-Cano, 1994). It should also be noted that the TG extract is able to induce plasmin generation on its own.

Proteomic analysis of the TG extract allowed us to identify 10 proteins that act as ligands for plasminogen. Since the genome of *S. bovis* has not been sequenced, the identification of these proteins has been possible by cross-species comparison with other schistosomes whose genomes have been wholly (*S. mansoni*) or partly (*S. japonicum*) sequenced (Wilson et al., 2004; Barret et al., 2005).

Orthologues of all this proteins identified in *S. bovis*, except the two hypothetical proteins of *S. japonicum* (AAW24823 and AAP06049, Table 1), have been identified in the tegument of *S. mansoni* (van Balkom et al., 2005; Braschi et al., 2006) and/or *S. japonicum* (Liu et al., 2006).

The most prominent spots on the 2D ligand blots corresponded to isoforms of enolase, GAPDH and actin. Of these proteins, enolase is the one that has been best characterised as a plasminogen receptor.

Enolase is a multifunctional glycolytic protein that belongs to a novel class of surface proteins that do not possess classical machinery for surface transport and yet are transported on the cell surface through an unknown mechanism (Pancholi, 2001). Enolase has been characterized as a plasminogen-binding protein on the surface of several pathogens, and this interaction with plasminogen has been shown to induce fibrinolytic activity and to be involved in invasiveness within the host (Bergmann et al., 2001; Jong et al., 2003). In helminths it has also been identified as a plasminogen-binding protein in the excretion-secretion products of *Fasciola hepatica* and in several tissues of *Onchocerca volvulus* (Jolodan et al., 2003; Bernal et al., 2004). In the TG of *S. bovis*, we identified 8 plasminogen-binding isoforms of enolase. In recent studies it has been shown that the four isoforms showing the same MWs and different pIs (spots 3, 4, 5 and 6) are recognised by the sera of sheep infected with *S. bovis*, while the other four isoforms are not antigenic and differ in both their MWs and their pIs (spots 7, 11, 13 and 14) (Pérez-Sánchez et al., 2006).

GAPDH is also a glycolytic enzyme and a non-classic surface protein of several bacterial species. GAPDH is a major plasminogen-binding protein in bacterial and fungal cells (Crowe et al., 2003; Bergmann et al., 2004). A recombinant GAPDH form from the filarial parasite *Onchocerca volvulus* has also been reported to bind plasminogen (Erttmann et al., 2005). In the tegument of *S. bovis*, we identified 3 isoforms of GAPDH that bind plasminogen, of which only 2 were antigenic (spots 26 and 27), as has been described recently (Pérez-Sánchez et al., 2006).

According to Braschi et al. (2006), the presence of glycolytic enzymes –such as enolase and GAPDH- in the tegument of the schistosomes could utilise incoming glucose for ATP production required for functions such as nutrient or solute uptake, or for changes in cytoskeletal conformation.

Actin, one of the most abundant cellular proteins, is found in all eukaryotic cells and for some time it has been known to bind to plasminogen kringle regions and to accelerate plasmin generation by t-PA (Lind and Smith, 1991). In schistosomes, it forms part of the myofibrils of the tegument and it is the major component of the spines of the surface of the worms (Jones et al., 2004). In *S. bovis*, we detected four isoforms of actin that bound plasminogen and, as has been reported recently (Pérez-Sánchez et al., 2006), they were not recognised by the host immune system. In that same work, we also reported that the tegument of *S. bovis* contains another two isoforms of actin that, in contrast, are antigenic but that, according to the present results, do not bind plasminogen.

This multiplicity of isoforms of enolase, GAPDH and actin, some of which are not antigenic, suggests that *S. bovis* could use paralogues of essential enzymes when they are targeted by the immune system in order to prevent loss of function, in a similar way to that suggested for *S. mansoni* (Verjovski-Almeida et al., 2003).

Other proteins identified in this study were ATP:guanidino kinase, triose-phosphate isomerase, adenylate kinase, fructose biphosphate aldolase, and phosphoglycerate mutase. The latter two have also been identified in the wall of *Candida albicans*, where they bind and

enhance the activation of plasminogen (Crowe et al., 2003). Regarding the rest of the proteins identified, this is the first time they have been reported to bind plasminogen.

This study represents the first report about the interactions of plasminogen with schistosome proteins. We demonstrate that plasminogen binds to the tegument surface of male *S. bovis* worms and that a protein extract of the tegument has the capacity to initiate plasmin generation and to enhance plasmin generation by t-PA. These observations are consistent with the hypothesis that male *S. bovis* worms would use host plasminogen to prevent blood clotting around them.

We have also identified a range of proteins from the tegument of *S. bovis* adult worms that bind plasminogen. We are unaware of which of these proteins might have biological significance as plasminogen receptors *in vivo*. This role requires their expression on the outer tegument surface of the worm. Consequently, further investigation will be required to determine which of these proteins are exposed to the host blood and to establish the physiological role of their interaction with plasminogen.

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Figure legends

Fig. 1. Plasminogen binding to 1 μg of TG extract of *Schistosoma bovis* measured over a range of plasminogen amounts using a microtitre plate method. (♦) Incubation with increasing amounts of plasminogen, 0-3 μg . (■) Competition assay: 40 mM ϵACA was included during the incubation with plasminogen. (▲) Control wells without TG, coated only with BSA. Each point is the mean of three replicates $\pm$ SD. The assay is typical of four experiments.

Fig. 2. SDS-PAGE of the TG extract and ligand blotting. Lane 1: silver staining of TG proteins. Lane 2: ligand blot with 35 $\mu\text{g}/\text{ml}$ of plasminogen. Lane 3: control ligand blot without TG. Lane 4: control ligand blot without plasminogen.

Fig. 3. Two-dimensional images of the TG extract from adult *Schistosoma bovis* worms. (A) and (B) silver-stained gels of 12% polyacrylamide and pH ranges of 5-8 and 7-10. (C) and (D) plasminogen-binding spots revealed on ligand blots from gels A and B. Numbers indicate the spots analysed by mass spectrometry.

Fig. 4. Activation of plasminogen in the presence of tegumental proteins of adult *Schistosoma bovis* worms. (□) 10 nM t-PA was added to mixtures containing 5 μg of plasminogen, 0.6 mM S2251, and increasing amounts of TG proteins (0-12 μg). (■) No t-PA was added to reaction mixtures. Each point is the mean of three replicates $\pm$ SD.

Fig. 5. Plasminogen binding on the surface of adult *S. bovis* worms as shown by immunofluorescence staining and confocal microscopy. Panels A-C are projected images derived from sections generated by the confocal microscope; panels D-F are confocal microscope sections from the middle plane of each specimen; panels G-I are the corresponding transmitted light images of panels D-F. Panels C and F show the results of the competition

experiments: the addition of 40 mM ϵ ACA during the plasminogen incubation step completely abolished plasminogen binding. ae, anterior end; f, female; m, male; os, oral sucker; pe, posterior end.

Table legend

Table 1. Identification of plasminogen binding proteins of the TG by MALDI-TOF-TOF mass spectrometry and NCBI nr database searching. Cover. %, sequence coverage; exp, experimental; theo, theoretical.

Table 1

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Cover. %, sequence coverage; exp, experimental; theo, theoretical.

Spot	Protein	Species	Accession No. (NCBI)	MW (kDa) Theo/exp	pI Theo/exp	Cover. %	MASCOT score
1	Hypothetical protein ^a	<i>S. japonicum</i>	AAW24823	60/68	6.8/6.3	4	120
3	Enolase ^a	<i>S. mansoni</i>	AAC46886	47/56	6.2/6.7	22	90
4	Enolase	<i>S. mansoni</i>	AAC46886	47/56	6.2/6.8	14	97
5	Enolase	<i>S. mansoni</i>	AAC46886	47/56	6.2/6.9	16	86
6	Enolase	<i>S. japonicum</i>	P33676	48/56	6.2/7.1	24	125
7	Enolase ^a	<i>S. mansoni</i>	AAC46886	47/52	6.2/6.7	12	103
8	Actin	<i>S. mansoni</i>	AAA62377	42/50	5.5/5.9	29	172
9	Actin	<i>S. mansoni</i>	AAA62377	42/48	5.5/6.3	29	152
10	Actin	<i>S. mansoni</i>	AAA62377	42/45	5.5/6.1	23	95
11	Enolase ^a	<i>S. mansoni</i>	AAC46886	47/42	6.2/6.1	9	132
12	Actin	<i>S. mansoni</i>	AAA62377	42/46	5.5/6.4	18	113
13	Enolase ^b	<i>S. mansoni</i>	AAC46886	47/48	6.2/6.6	-	61
14	Enolase ^b	<i>S. mansoni</i>	AAC46886	47/48	6.2/6.7	-	82
15	Glyceraldehyde-3-phosphate dehydrogenase ^b	<i>S. mansoni</i>	JL0121	37/43	7.7/7.1	-	107
16	Hypothetical (Clone ZZD114 mRNA sequence) ^a	<i>S. japonicum</i>	AAP06049	33/36	6.5/6.8	12	97
17	ATP: guanidino kinase	<i>S. mansoni</i>	P16641	77/82	8.1/7.7	19	120
18	ATP: guanidino kinase	<i>S. mansoni</i>	P16641	77/82	8.1/7.8	34	208
19	ATP: guanidino kinase	<i>S. mansoni</i>	P16641	77/82	8.1/7.9	12	112
25	Fructose 1,6 bisphosphate aldolase	<i>S. mansoni</i>	AAA57567	40/46	7.4/7.8	49	225
26	Glyceraldehyde-3-phosphate dehydrogenase	<i>S. mansoni</i>	JL0121	37/44	7.7/7.9	21	90
27	Glyceraldehyde-3-phosphate dehydrogenase ^a	<i>S. mansoni</i>	JL0121	37/44	7.7/8.6	17	134
28	Phosphoglycerate mutase ^a	<i>S. japonicum</i>	AAL30898	28/32	7.5/7.7	16	128
29	Triose-phosphate isomerase	<i>S. mansoni</i>	A38233	28/32	7.4/7.8	37	163
30	Adenylate kinase ^a	<i>S. mansoni</i>	P25824	23/29	8.8/8.4	35	134

^a Spots identified using PMF data in combination with MS/MS data.^b Spots unidentified using PMF data but identified using MS/MS data.

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3.

Figure 4

Figure 4

Figure 5

Figure 5

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June 19, 2006

Dr. J. Horton
Experimental Parasitology, Editor.

Dear Editor:

Please allow me to start by thanking you for your letter and the opportunity it brings to publish our manuscript in Experimental Parasitology. We would also like to acknowledge your feedback and that of the reviewers involved, which has been of great value to us.

According to your suggestions we have changed the manuscript title, which now stands as follows: "*Schistosoma bovis*: plasminogen binding in adults and the identification of plasminogen-binding proteins from the worm tegument. We also provided the references list in the Experimental Parasitology format. Please, let us apologize for this mistake.

We have studied the reviewers' comments thoroughly and have now revised the manuscript bearing in mind all the points and suggestions made that can be read in the "response to reviews".

We hope that with the amendments made, the revised manuscript can be now published in Experimental Parasitology.

Yours faithfully,

Dr. Ana Oleaga